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Influence of the Amino Acid Differences Between the Hemagglutinin HA1 Domains of Influenza Virus H1N1 Strains on Their Reaction With Antibody

Joachim Drescher* and Roman Aron
Hannover Medical School, Institute of Virology, Hannover, Germany

For influenza H1N1 strains, including some of their escape variants, the association of amino acid differences located at their hemagglutinin HA1 domains with their antigenic relationship was examined. The antigenic relationship was recorded in terms of the ratios of hemagglutination inhibition (HI) titers, the concentration of antibody molecules recognized by the virus, and the equilibrium constant of epitope–paratope interaction determined with heterologous virus compared to that found with homologous virus. The HI titers of antisera were found to depend primarily on the concentration of antibody molecules recognized by the virus and much less on the equilibrium constants. The avidity of antibody in sera raised against historically later strains with earlier strains was higher than vice versa. In contrast to the results obtained with antisera, the same concentration of monoclonal antibody directed to the Sb site of A/Brazil virus was recognized by both heterologous and homologous viruses, and the differences in HI titers observed were due to avidity changes only. Some of the amino acid differences located at each of the antigenic sites were found to be associated with a reduction in the HI titers and in the concentration of antibody molecules recognized by heterologous virus, whereas other differences in addition decreased the avidity of epitope–paratope interaction. Further amino acid differences decreased the avidity only. The strains tested differed also in their amino acids located outside the antigenic sites. However, an influence of these differences on the reaction of virus with antibody could not be evidenced. For the strains tested, the antigenic hemagglutinin drift occurred by reduction of the concentration of antibody molecules recognized by the virus and by avidity changes, which, in turn, were caused by exchanges of some key residues located at the antigenic sites. ***J. Med. Virol. 57: 397–404, 1999.*** © 1999 Wiley-Liss, Inc.

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INTRODUCTION

The hemagglutinin (HA) is the major surface antigen of influenza viruses and its HA1 domain shows frequent antigenic variation of a major (antigenic shift) or a minor (antigenic drift) kind [Murphy and Webster, 1996]. The amino acid changes in field strains caused by antigenic drift [Ward, 1981; Both et al., 1983] and in escape variants selected by monoclonal antibody [Webster and Laver, 1980; Laver et al., 1981; Daniels et al., 1983; Newton et al., 1983] have been found to cluster in distinct regions of the HA1 domain of the HA molecule, referred to as antigenic sites.

Five antigenic sites (designated A through E) have been described for H3 hemagglutinin [Wiley et al., 1981; Daniels et al., 1983; Wiley and Skehel, 1987] and four sites (designated Sa, Sb, Ca and Cb) in H1 hemagglutinin [Breschkin et al., 1981; Gerhard et al., 1981; Jackson et al., 1982]. The H1 site Ca has been further divided into the subsites Ca1 and Ca2 [Caton et al., 1982]. The H1 and H3 sites are at least partially equivalent (e.g., the H1 site Sb to the H3 site B, Caton et al., 1982; Yewdell et al., 1983).

Antigenic drift in influenza hemagglutinin is thought to occur by the sequential accumulation of point mutations in the HA gene, which change the amino acid sequence of antigenic sites and alter their reactivity with antibody [Murphy and Webster, 1996]. Although the HA genes of many strains have been sequenced, it has not been possible to determine precisely which of these sequence differences were responsible for the variation in the antigenicity of the HA [Air and Laver, 1986]. By comparing the amino acid changes between related strains and escape variants with their

*Correspondence to: Professor Dr. J. Drescher, Institute of Virology, Hannover Medical School, Carl-Neuberg-Str. 1, D 30623 Hannover, Germany.

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reaction to monoclonal antibody, amino acid residues have been identified that are responsible for the integrity of the respective epitopes [Both et al., 1983; Nakajima et al., 1983; Air and Laver, 1986; Brown et al., 1990). The hemagglutinin drift can be quantitated in terms of the ratio of HI titers with heterologous virus to that of HI titers with homologous virus [Murphy and Webster, 1996].

However, HI titers depend on both the concentration of antibody molecules (A) and the equilibrium constant (K) of epitope–paratope interaction [Kuschel and Drescher, 1978]. Therefore, a complete analysis of the association between amino acid exchanges and the reactivity of viruses with antibody requires that the association with A and K should also be determined.

The availability of a method for determining the A and K values for the reaction with both homologous and heterologous viruses [Drescher et al., 1990] prompted an investigation into the influence of amino acid differences between the HA1 domains of a series of influenza H1N1 virus strains, including escape variants, on the HI titers, and on A and K values. The values determined with heterologous virus were divided by the respective values obtained for homologous virus and the association between these ratios and amino acid differences was examined. In addition to viral antisera, monoclonal antibodies directed to the Sb site of A/Brazil virus were tested in a similar manner.

MATERIAL AND METHODS

Virus

The following strains of egg-adapted influenza virus were used: A/FM/1/47 (H1N1), A/PR/301/54 (H1N1), A/USSR/97/77 (H1N1), and A/Brazil/11/78 (H1N1). In addition, the A/Brazil escape variants EV-Sa (with altered site Sa) and EV-Sb (with altered site Sb) of A/Brazil/11/78, which were produced and tested as described previously [Drescher and Verhagen, 1993], and the reassortant A/Aichi/2/68 (H3)–A/Bel/42/41 (N1) were used. Virus was purified by means of sucrose gradient ultracentrifugation [Laver, 1960]. The hemagglutinin titers were determined by means of the HA pattern test [Palmer et al., 1975].

Antibody

Antisera against the strains of virus listed above were raised in chickens. Each animal received two 1-ml doses (1,000 HA, the first with and the second without incomplete Freund's adjuvant) intramuscularly at intervals of 10 days. Blood samples were drawn by cardiac puncture at graded time intervals. Sera were pretreated with M/90 KIO_4 [Dowdle et al., 1979]. In control experiments, it was found that pretreatment with receptor destroying enzyme (RDE) [Masurel, 1969] yielded analogous results. Sera were preadsorbed onto an excess of the reassortant A/Aichi/2/68 (H3)–A/Bel/42/41 (N1) to remove neuraminidase antibody. Monoclonal antibodies directed against A/Brazil were produced and tested as described previously [Drescher et al., 1987]. HI antibody titers were determined as described by Palmer et al. [1975] and were expressed in terms of the reciprocal highest antibody dilution yielding complete inhibition of four agglutinating doses of virus.

Determination of the Concentration of Antibody Molecules (A), the Equilibrium Constant of Paratope–Epitope Interaction (K), and the Number of Epitopes Recognized per Virus Particle by Antibody(s)

The values of A, K, and s were determined as described previously [Drescher et al., 1987], using homologous and heterologous virus. The K values were calculated as defined by Fazekas de St. Groth and Webster [1961] as ratios of the product of the concentration of free epitopes and paratopes to the concentration of epitope–paratope complexes (cgs units). As a consequence, high values of K relate to low avidity and vice versa. The antisera used were found to yield s values not differing significantly from 1,600 when allowed to react with homologous virus, indicating that they recognized within experimental limits all of the hemagglutinin epitopes present [Drescher and Verhagen, 1993].

Ratios Used for Comparing Homologous and Heterologous Virus Antibody Interactions

The reaction of antibody with homologous and heterologous virus was compared in terms of the following ratios:

(i) HI_r = heterologous HI titer divided by homologous HI titer.
(ii) A_r = concentration of antibody molecules recognized by heterologous virus divided by concentration of antibody molecules recognized by homologous virus.
(iii) K_r = K value obtained with heterologous virus divided by K value obtained with homologous virus.

These ratios were presented in terms of their geometric means (± SD or SE).

Sequencing of Viral RNA

Viral RNA was extracted as described by Palese and Schulman [1976] and was sequenced directly by the didesoxy chain-termination technique [Sanger et al., 1977], using avian myeloblastosis virus-reverse transcriptase (AMV-RT). The primers used for the HA1 gene were complementary to the viral RNA at positions 1–15 (AGC AAA AGC AGG GGA), 157–171 (AGT GAC ACA CTC TG), 305–318 (ATC ATG GTC CTA C), 506–520 (AAG CAG TTT TTA CAG), 668–682 (AAA TGC TTA TGT CTC), and 875–887 (CAT CAT CAC CTC A). The primers were synthesized by MWG-Biotech (Ebersberg, Germany). The reaction mixture contained the annealing mixture, AMV-RT buffer (50 mM Tris-HCl, pH 8.3, KCl 50 mM, $MgCl_2$ 10 mM, DTT 10 mM, 0.5 mM spermidine), labeling mix (dGTP 1.5 μM, dCTP 1.5 μM, dTTP 1.5 μM), (-^{35}S) dATP 10 μCi (1,000 Ci/

mmole), (Amersham), and 4 units of Rnase-inhibitor, (Boehringer Mannheim, Germany). The reaction was started by the addition of 4–8 units of AMV-RT (Promega) and the mixture was incubated at 42°C for 2–5 min. After the labeling, 4-μl aliquots of each of the labeling mixtures were added to the chain-termination tubes: ddATP (160 μM dATP, 40 μM ddATP, 200 μM dGTP, 200 μM dCTP), ddCTP (160 μM dCTP, 40 μM ddCTP, 200 μM dATP, 200 μM dGTP, 200 μM dTTP), ddGTP (160 μM dGTP, 40 μM ddGTP, 200 μM dATP, 200 μM dCTP, 200 μM dTTP), and ddTTP (160 μM dTTP, 40 μM ddTTP, 200 μM dATP, 200 μM dGTP, 200 μM dCTP) and incubated at 42°C for 15 min. A cold-chase solution containing 1 mM of each nucleotide was added and incubated for a further 30 min at 42°C. Terminal deoxynucleotidyl transferase 4 units (Promega) were added to the chase mixture when necessary to resolve sequence ambiguities [De Borde et al., 1986]. Reactions were terminated by the addition of 3 μl of the stop solution and concentrated for 10 min in a Speed Vac concentrator. Denaturation was carried out by heating at 95°C for 3 min before gel electrophoresis. Acrylamide slab gels (17 + 50 + 0.019 cm) were prepared in 100 mM Tris-borate, 1 mM ethylenediamine tetraacetic acid (EDTA) buffer, pH 8.3. Gels were fixed for 15 min in 10% (w/v) acetic acid, dried, and exposed with a Kodak X-Omat film for 1–5 days.

Description of Amino Acid Differences

The term amino acid difference was used to indicate the presence of different amino acids at the same position in the two viruses compared. The abbreviation for the amino acid present in the virus allowed to react with antibody is given first followed by the symbol of the amino acid present in the virus used for raising antibody. For example, when A/FM/1/47 virus was allowed to react with A/PR/301/54 antibody, the amino acid difference at position 157 was scored as $(T/K)^{157}$, whereas the symbol $(K/T)^{157}$ was used to describe the amino acid difference at position 157 for the reaction of A/PR/301/54 virus with A/FM/1/47 antibody.

The amino acids were divided into the following groups: acidic hydrophilic, neutral, basic hydrophilic, and hydrophobic. If the differing amino acids belonged to the same group, the difference was designated as "within equal group" and the term "difference between unequal groups" was used to denote differences in which the amino acids belonged to different groups.

Statistical Analysis

The ratios HI_r, A_r, and K_r were tested for significant difference from 1.0 using Student's *t*-test and the logarithms of values. The relationship between Hi_r, A_r, and K_r was tested by multiple regression. The association between amino acid differences and HI_r, A_r and K_r was examined as follows:

(i) The HI_r, A_r, and K_r ratios were calculated separately for reactions with and without a given amino acid difference and the mean values of the corresponding ratios were tested for significant differences by means of the Mann–Whitney test.

(ii) The association between the amino acid differences and each of the ratios HI_r, A_r, and K_r was tested by means of discriminant analysis, using as binary outcome variable the presence or absence of ratios above or below a limiting value. For example, all HI_r ratios > 0.5 were placed into one group and all HI_r ratios < 0.5 into a second group, when using 0.5 as limiting value.

All tests were performed with the number crunching statistical system (NCSS) program. P values < .05 were considered significant, unless otherwise indicated.

RESULTS

Amino Acid Sequence of the HA1 Domain of the Viruses Tested

The HA1 genes of the viruses tested were sequenced and the amino acid sequences deduced. The results obtained are given in Table I for amino acid positions where at least one amino acid difference was recorded. Residues were numbered with reference to the protein sequence alignment of A/Aichi/2/68 and A/PR8/34. When additional residues occurred in H1N1 strains relative to A/Aichi/2/68, they were numbered according to the preceding residue and a subscript (e.g., 125c). The amino acid positions belonging to the antigenic sites Sa through Cb were assigned as described by Caton et al. [1982] and Raymond et al. [1983, 1986].

With two exceptions (residue no. 138: A instead of S and residue 157: K instead of E), the amino acid sequence found for the strain A/Brazil/11/78 was the same as described by Raymond et al. [1986]. The difference found was probably caused by laboratory drift due to passing of virus since isolation.

When comparing the amino acid differences between test viruses without escape variants, the largest number of differences was found outside the antigenic sites. Of the antigenic sites, Ca showed the largest and Sa the smallest number of differences. The escape variants EV-Sa and EV-Sb differed by only one amino acid at sites Sa and Sb, respectively, from their parent virus (A/Brazil) and the strain A/PR301 was found to have the same Sa and Sb site as EV-Sb.

Serologic Relationship Between Test Viruses

The reactions of test viruses with antisera against homologous and heterologous virus were analyzed in terms of the ratios HI_r (HI titer against heterologous virus divided by HI titer against homologous virus), A_r (concentration of antibody molecules recognized by heterologous virus divided by concentration of antibody molecules recognized by homologous virus), and K_r (equilibrium constant measured with heterologous virus divided by value obtained with homologous virus). The geometric means of these ratios are given in Table II. By multiple regression analysis, the HI_r values were found to depend much more on A_r than on K_r [regres-

TABLE I. Amino Acid Differences Between Test Viruses

Amino acid position no.	Antigenic site	Amino acids present on virus[a]					
		A/FM/1/47	A/PR/301/54	A/USSR/97/77	A/Brazil/11/78	A/Brazil EV-Sa	A/Brazil EV-Sb
125c	Sa	R	R	K	R	R	R
157		T	K	K	K	E	K
192	Sb	R	K	K	K	K	K
193		N	T	T	T	T	T
194		L	I	I	I	I	I
196		R	Q	R	R	R	Q
142	Ca	A	A	K	K	K	K
143		G	R	G	G	G	G
172		Q	K	K	K	K	K
219		E	K	E	K	K	K
225		G	N	G	G	G	G
227		A	P	A	E	E	E
79	Cb	L	L	V	F	F	F
81		K	N	K	K	K	K
82		R	R	K	K	K	K
65	None	N	N	N	S	S	S
101		D	D	Y	Y	Y	Y
132		I	T	V	I	I	I
133		T	I	T	T	T	T
138		A	A	S	A	A	A
149		K	K	R	R	R	R
200		A	A	T	A	A	A
230		M	M	I	I	I	I
248		N	T	D	N	N	N
256		H	H	H	Y	Y	Y
260a		N	N	N	S	S	S
264		F	P	F	F	F	F
297		I	I	I	V	V	V
310		K	K	R	R	R	R

[a]Only amino acids differing between at least two strains were given.
A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.

sion coefficient for HI_r versus A_r 1.006 ($P < .05$) and for HI_r versus K_r −0.0324 ($P < .05$)].

The avidity of the reaction of virus with antibody directed to viruses isolated previously (e.g., A/Brazil virus with A/FM1 antibody) decreased regularly as indicated by increased K_r values. In contrast, the avidity of the reaction of antibody to more recently isolated viruses with preceding strains (e.g., A/Brazil antibody with A/FM1 virus) remained either unchanged or even increased.

Influence of Amino Acid Differences Between Test Viruses on Values of HI_r, A_r, and K_r

The association of amino acid differences between the viruses allowed to react with antibody and the viruses used for raising antibody with the values of HI_r, A_r, and K_r was tested by discriminant analysis, using as binary outcome variables values below and above a limiting value (HI_r and A_r: 0.365 and 0.5; K_r: 0.7 and 1.5). In addition, the means of HI_r, A_r, and K_r calculated for reactions with and without a given amino acid difference were tested for significant differences.

The amino acid differences located within the antigenic sites that were found to be significantly ($P < .05$) associated with HI_r, A_r, and/or K_r by discriminant analysis are summarized in Table III. The respective discriminant analyses yielded a reduction in classification error over that expected for random classification of more than 88.3%.

Amino acid differences found by discriminant analysis to be associated with HI_r, A_r, and/or K_r also yielded significantly different ($P < .05$) mean values of the respective ratios when comparing reactions with and without these amino acid differences.

The following amino acid differences occurred always in combination: $(A/K)^{142}$ and $(R/K)^{82}$, $(K/A)^{142}$ and $(K/R)^{82}$, $(N/G)^{225}$ and $(R/G)^{143}$, and $(R/K)^{192}$, $(N/T)^{193}$, $(L/J)^{194}$ and $(Q/K)^{172}$. As a consequence, no conclusion was reached on whether all of the differences occurring in combination were associated with the ratios HI_r, A_r, and K_r.

The amino acid differences found to be associated with HI_r, A_r, and K_r related both to differences within the same group and to differences between different amino acid groups. The amino acid differences were divided into the following groups according to the association found (Table III):

(i) differences associated with decreased HI_r and A_r only;

TABLE II. Ratios of HI_r, A_r, and K_r for Virus Antibody Interactions

Virus allowed to react with antibody (=virus1)	Antibody raised against virus (=virus2)	Geometric means of ratio[b]		
		HI_r	A_r	K_r
A/FM1[a]	A/USSR	0.210+*	0.250+	0.891–
A/USSR	A/FM1	0.180+	0.280+	1.605+
A/FM1[a]	A/Brazil	0.321+	0.280+	1.021–
A/Brazil	A/FM1	0.172+	0.260+	1.705+
A/FM1[a]	EV-Sa	0.450+	0.490+	1.045–
EV-Sa	A/FM1	0.132+	0.190+	1.280–
A/FM1[a]	EV-Sb	0.320+	0.280+	0.566+
EV-Sb	A/FM1	0.115+	0.188+	1.583+
PR301[a]	A/USSR	0.174+	0.120+	0.638–
A/USSR	A/PR301	0.109+	0.210+	4.315+
PR301[a]	A/Brazil	0.183+	0.195+	0.593+
A/Brazil	A/PR301	0.088+	0.018+	3.512+
A/PR301[a]	EV-Sa	0.290+	0.350+	0.871–
EV-Sa	A/PR301	0.079+	0.15+	1.830+
A/PR301[a]	EV-Sb	0.270+	0.250+	1.021–
EV-Sb	A/PR301	0.039+	0.125+	1.531+
A/FM1[a]	A/PR301	0.216+	0.266+	1.162–
A/PR301	A/FM1	0.125+	0.257+	1.850+
A/USSR[a]	A/Brazil	0.721+	0.71(+)	1.271–
A/Brazil	A/USSR	0.695+	0.737(+)	1.974+
A/USSR[a]	EV-Sa	0.740+	0.780–	0.725–
EV-Sa	A/USSR	0.330+	0.440+	1.490+
A/USSR[a]	EV-Sb	0.810{+}**	0.750–	0.640–
EV-Sb	A/USSR	0.360+	0.445+	1.662+
A/Brazil	EV-Sa	0.830–	0.840–	1.293–
EV-Sa	A/Brazil	0.670+	0.780–	0.566+
A/Brazil	EV-Sb	0.920–***	0.830–	0.571+
EV-Sb	A/Brazil	0.750–	0.690+	0.752–
EV-Sa	EV-Sb	0.710+	0.750–	0.405+
EV-Sb	EV-Sa	0.510–	0.700(+)	3.395+

[a]Virus 1 isolated before virus 2.
[b]Mean values of 10 to 15 determinations. The SE of the arithmetic means of the logarithms of values did not exceed the following limits: HI_r: 0.078; A_r: 0.085, and K_r: 0.097.
*+Value significantly differing from 1.0 ($P < .05$).
**(+)Value significantly differing from 1.0 ($P > 0.5$; <.10).
***Value not significantly differing from 1.0 ($P > .11$).

(ii) differences associated with decreased HI_r and A_r, and decreased avidity (increased K_r values);
(iii) differences associated with decreased avidity only.

Furthermore, a number of amino acid differences were without detectable influence on HI_r, A_r, and K_r.

Due to the small number of reactions for which an increased avidity (K_r significantly smaller than 1.0) was observed, no meaningful discriminant analysis was achieved for these values. However, some amino acid differences were identified for which the respective average of K_r was significantly smaller than for reactions without these differences [$(R/K)^{192}$, $(N/T)^{193}$, $(L/J)^{194}$, $(A/K)^{142}$, $(Q/K)^{172}$, $(A/E)^{227}$, $(L/F)^{79}$, and $(R/K)^{82}$].

For the amino acid differences located outside the antigenic sites, no association with HI_r, A_r, and K_r was found, with the exception of differences that occurred in combination with differences located within antigenic sites and associated with one of these ratios (see Table III). For the latter, an association could not be ruled out by means of discriminant analyses. However, as regards differences represented by buried residues, it seems unlikely that these influenced the epitope–paratope interaction.

Table IV gives the results obtained when testing in like manner the total number of amino acid differences at indicated antigenic sites and outside the antigenic sites. The differences per antigenic site were further divided into exchanges of amino acids within the same or between different groups of amino acids. The differences located outside the antigenic sites were divided further into residues located in a surface position and buried residues. Note that the changes at sites S_b and C_b were associated with HI_r and A_r and that no influence of amino acid differences located outside the antigenic sites on the ratios tested could be detected by discriminant analysis.

Reaction of Monoclonal A/Brazil Antibody Directed to Site Sb With Test Viruses

Monoclonal antibody directed to site Sb of A/Brazil virus was allowed to react with the viruses employed. The HI, A, and K values found with heterologous viruses were divided into the respective values found with A/Brazil (= ratios HI_r, A_r, and K_r) and were tested for significant differences from 1 using a *t*-test. Representative examples of the results obtained are given in Table V.

The A_r values found did not differ significantly from 1.0, indicating that the test viruses recognized all of the antibody molecules within experimental limits. The HI titers were significantly increased for reactions with increased avidity (reactions with EV-Sa, A/FM1, and A/PR301).

The Sb antibody combined with higher avidity with EV-Sa than with A/Brazil. Because EV-Sa and A/Brazil differ only by $(E/K)^{157}$, site Sa, it is reasonable to assume that this difference was associated with the avidity increase observed.

No avidity difference was recorded when the Sb antibody was allowed to react with A/USSR and A/Brazil. This finding indicates that the amino acid differences between these viruses [$(K/R)^{125c}$, site Sa, $(E/K)^{219}$, site Ca, $(A/E)^{227}$, site Ca, and $(V/F)^{79}$, site Cb] were not associated with increased avidity.

The strains A/FM1 and A/PR301 both reacted with increased avidity with monoclonal Sb antibody. Both strains differed from A/Brazil by $(A/K)^{142}$, $(L/F)^{79}$, and $(R/K)^{82}$, and reactions with these differences have been found to yield significantly lower mean values of K_r (i.e., increased avidity) for antisera than reactions without such differences. Due to the limited number of different virus–antibody interactions available, no conclusions based on discriminant analysis and also including amino acid differences outside the antigenic sites could be reached.

TABLE III. Summary of Amino Acid Differences Found by Discriminant Analysis to Be Associated With Ratios HI_r, A_r, and/or K_r

Amino acid difference associated with		Amino acid difference[a] at antigenic site			
HI_r and A_r	K_r	Sa	Sb	Ca	Cb
Decrease	No change	(T/K)^{157}	(R/K)^{192} (L/J)^{194} (N/T)^{193}	(A/K)^{142} (+)[b] (Q/K)^{172} (N/G)^{225} (+++)[b] (R/G)^{143} (+++)[b]	(N/K)^{81} (+++)[b] (R/K)^{82} (+)[b]
Decrease	Increase			(K/A)^{142} (++)[b]	(K.R)^{82} (++)[b]
No change	Increase	(K/T)^{157}			(F/V)^{79}

[a]Amino acid on virus allowed to react with antibody/amino acid on virus used for raising antibody.
[b]Occurring in combination with the following amino acid differences located outside the antigenic sites:
+(M/I)^{230} (buried), (D/I)^{101} (buried), (K/R)^{149c} and (K/R)^{310};
++(I/M)^{230} (buried), (I/D)^{101} (buried), (R/K)^{149c} and (R/K)^{310};
+++(J/T)^{133} and (P/F)^{264} (buried).

TABLE IV. Association of Amino Acid Differences With HI_r, A_r, and K_r

Antigenic site	Amino acid difference within equal or unequal group	HI_r			A_r			K_r		
		Mean value	Discriminant analysis <0.36[a]	<0.5[d]	Mean value	Discriminant analysis <0.36[d]	<0.5[d]	Mean analysis	Discriminant analysis <0.7[d]	>1.6[d]
Sa	Equal[a]	–	–	–	–	–	–	–	–	–
	Unequal[b]	(+)[c]	–	–	–	–	–	–	–	–
Sb	Equal	+[c]	+*	+	+	+	+	–	–	–
	Unequal	–	–	–	(+)*	–	–	–	–	–
Ca	Equal	+	–	–	+	–	–	–	–	–
	Unequal	+	+	–	+	+	–	–	–	–
Cb	Equal	+	+	+	+	+	+	–	–	–
	Unequal	+	+	+	+	+	+	–	–	–
Sa	Both	(+)	–	–	–	–	–	–	–	–
Sb	Both	+	+	+	+	+	+	–	–	–
Ca	Both	+	–	–	+	–	–	–	–	–
Cb	Both	+	+	+	+	+	+	–	–	–
All	Both	+	+	+	+	+	+	+	–	+
None, buried	Both	+	–	–	+	–	–	–	–	–
None, surface	Both	+	–	–	+	–	–	–	–	–
None	Both	+	–	–	+	–	–	–	–	–

[a]The differing amino acids belonged to the same group of amino acids.
[b]The differing amino acids belonged to different groups of amino acids.
[c]Mean value of ratios obtained for reactions with indicated amino acid difference significantly [+: $P < .05$; (+): $P < .1$, $>.05$] differing from mean value obtained for reactions without the indicated amino acid difference. –: No significant ($P > .1$) difference was found.
[d]Limiting value used for discriminant analysis.
*Significant (+: $P < .05$; (+): $P < .1$, $>.05$) association found.

TABLE V. Reaction of Monoclonal Brazil Sb Antibody With Antigenic Site Sb of Different Viruses

Viruses compared		A_r values compared[a]		K values compared[a]		HI values compared[a]	
Virus 1	Virus 2	A_r[b]	SD	K_r[c]	SD	HI_r[d]	SD
EV-Sa	Brazil	0.928	0.28	0.141*	0.07	2.00	0.816
USSR	Brazil	1.081	0.61	1.008	0.559	0.86	0.35
FM1	Brazil	0.816	0.31	0.393	0.538	4.00	1.20
PR301	Brazil	0.695	0.42	0.248	0.443	4.25	1.35

[a]Average of eight determinations.
[b]A_r = A value obtained with virus 1 divided by A value obtained with virus 2.
[c]K_r = K value obtained with virus 1 divided by K value obtained with virus 2.
[d]HI_r = HI titer obtained with virus 1 divided by HI titer obtained with virus 2.
*Underlined values differ significantly from 1.0 ($P < .05$, t test).

DISCUSSION

When analyzing the association of amino acid differences between the viruses tested with their antigenic relationship, a complete analysis will only be achieved if the antisera used recognize all the epitopes present in the homologous virus. This conclusion is obvious from the following consideration: if two viruses, differing only at one antigenic site, are allowed to react with an antiserum directed to one of them and the antiserum used does not recognize this antigenic site in the homologous virus, the influence of the amino acid difference at this site on the serologic results cannot be fully recognized. It has been described previously [Drescher et al., 1987] that the virus strains used possessed

about 1,600 hemagglutinin epitopes per virion (s). The antisera used yielded values of s not significantly differing from 1,600 when allowed to react with homologous virus. As a consequence, it was concluded that the antisera recognized within experimental limits all epitopes present in homologous virus, meeting the prerequisite outlined. Furthermore, the antisera used should yield ratios of HI_r, A_r, and K_r within a limited error range only. This condition was met by the antisera used in our experiments. However, no statement can be made about whether or not the use of different viruses, animals, or vaccination schedules would have given different results.

The antigenic relationship between the hemagglutinins of the H1N1 influenza viruses tested was expressed in terms of the ratio HI_r.. The HI_r values were found to depend primarily on the proportion of antibody molecules recognized by heterologous virus (A_r values) and much less on K_r values. Furthermore, the K_r values found indicated that the avidity of the reaction of antibody to previously isolated viruses with a more recently isolated virus regularly decreased in comparison to the avidity recorded for the homologous reaction. In contrast, the avidity of reaction of antibody to more recently isolated virus with preceding strains remained either unchanged or even increased.

Thus, the avidity of reaction of antibody against historically later strains with earlier strains was higher than vice versa, a finding fitting into the model described by Fazekas de St. Groth [1970] of a junior and senior relationship between influenza viruses.

The association of the ratios HI_r, A_r, and K_r with the amino acid differences between the virus allowed to react with antibody and the virus used for raising antibody was examined. By means of discriminant analysis, the conclusion was reached that some amino acid differences located at each antigenic site were associated with a decrease in HI_r and A_r only. Other differences, located at sites Ca and Cb, decreased HI_r, A_r, and also avidity. Two further differences located on sites Sa and Cb were associated with decreased avidity only.

Note that the avidity of epitope–paratope interaction was also found to be associated with the direction of amino acid differences: for example, the presence of K at position 142 of the virus allowed to react with antibody and of A at the same position of the virus used for raising antibody was associated with drastically reduced avidity, whereas the reverse difference (i.e., A at position 142 of the virus allowed to react with antibody and K at the same position of the virus used for raising antibody) failed to reduce avidity.

For the amino acid differences located outside the antigenic sites, no evidence of an association with HI_r, A_r, and K_r was obtained. However, for some of these differences an association could not be excluded because they occurred in combination with such differences located within the antigenic sites for which an association was found. However, the finding that the total number of differences located outside the antigenic regions was not associated with HI_r, A_r, and K_r argued against an influence of these differences on serology.

For the strains tested (excluding escape variants), the percentage of amino acid substitutions per year ranged from 0.16% (comparison of A/FM1 with A/USSR and of A/FM1 with A/Brazil) to 3.48% (comparison of A/USSR with A/Brazil) and was of the same magnitude as described for other influenza virus strains [Raymond et al., 1983]. The changes occurred at each of the antigenic sites and in addition, at 14 positions (9 buried and 5 located on the surface) located outside these antigenic sites; the analogous conclusion was reached by Raymond et al. [1983] for other H1N1 strains.

The groups of amino acids (acidic hydrophilic, neutral, basic hydrophilic, and hydrophobic) were examined to determine whether the differing acids influenced the association of amino acid differences with HI_r, A_r, and K_r. Thirty percent of the differences within antigenic sites were found to be associated with these ratios and 23.5% of all differences located at antigenic sites involved the exchange of a neutral amino acid against a basic hydrophilic amino acid. In contrast, these exchanges were not found for amino acids located outside the antigenic sites.

The results obtained with antisera reflect the reaction of viruses with antibodies of differing fine specificity. Therefore, in addition we examined the reaction of viruses with monoclonal antibody directed to the Sb site of A/Brazil virus in like manner. The results obtained differed from those described for antisera as follows:

(i) Although antisera recognized regularly fewer epitopes per virus particle (s) on heterologous than on homologous virus, the s values recorded for monoclonal antibody with homologous (A/Brazil) and heterologous viruses (A/FM1, A/PR301, A/USSR, EV-Sa) did not differ significantly.

(ii) The concentration of monoclonal antibody molecules recognized by heterologous viruses did not differ significantly from that recorded for homologous virus, whereas heterologous viruses recognized regularly less antibody molecules than homologous viruses when allowed to react with antisera.

The monoclonal antibody directed to the Sb site of A/Brazil showed higher avidity with EV-Sa than with A/Brazil. Because both viruses differ only by one residue at site Sa [$(E/K)^{157}$], it is obvious that an exchange of residues at a site not involved in epitope–paratope interaction altered the reactivity of another site, probably due to conformational change. It has been previously reported [Brown et al., 1990] that residues whose substitution inhibits binding of monoclonal antibody need not necessarily lie within the epitope recognized by the antibody.

Monoclonal Sb antibody directed to the Sb site of A/Brazil virus failed to combine with EV-Sb but reacted with A/PR301 virus. Because A/PR301 was found to have the same amino acid sequence at site Sb as did EV-Sb, this finding could be explained by assuming

that the Sb antibody combined with one of the antigenic sites Sa, Ca, or Cb on A/PR301. As an alternative explanation, it could be assumed that the amino acid differences between PR301 and EV-Sb, located at sites Sa, Ca, or Cb influenced the Sb site of this virus in such a way that it reacted with Sb antibody. It is not known at present which of these interpretations is correct.

Our results indicate that antigenic drift in the strains tested occurred by residue exchanges located at key positions within the antigenic sites, which, in turn, reduced the concentration of antibody molecules recognized by virus and/or the avidity of epitope–paratope interaction.

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